

3.)

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Setting up Allo- & Self restricted peptide CTL lines

BMC: L63 A2⁻ Allo

peptide L64 A2⁺ self

with 10, 235, 441 and C228 (true control).

Method: as left use DC from L64 as stimulator

Pro stimulate with peptide loaded CIRA2 cells (50 μ m).

Restimulated Σ

RNA-3 A2 (control)

CTL assay

Expand & cloning

CTL of clones

Transfer to 2 well to 48 well plate

fixed

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5

Setting up Self & Allo - control peptide specific
CTL line

PBMC L G 5 - Ant Self
L G 6 - Ant Allo

peptides: WT-1 126 187 280
+ve control E786-95

Methods, see previous page.

First round stimulation with T2 loaded peptides.

2. Restimulate with peptide loaded - CIRA2

4/2 - RMA-S - A2...

3/2 - Restimulation - CIRA2

2/3 - CTL

7/3 - Cloning

23/3 - CTL of clones

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Protocol for peptide specific allorestricted human CTL

1. Prepare stimulator cells: in case of T2 stimulators preincubate o/n 10^6 cells/ml/well in x24 well plates in RPMI medium plus 5% boiled (10 min) FCS with 100 nM peptide. If allo DC are used as stimulators, allo DC from A2 +ve individuals can be separated as following:

Incubate A2+ PBMC at 37°C O/N, harvest nonadherent cells, resuspend in RPMI+9%FCS at 5×10^6 /ml and layer onto metrizadime gradient carefully, spin at 1500 for 10 min and harvest the interface (30% low density DC, 20% recovered from PBMC counts). Wash X3 with medium, resuspend at 10^6 /ml, add peptides to a final conc of 50uM and incubate at 37°C for 2 hr. (3000 rad) The stimulator can be added to responders directly w/o washing off peptides.

2. Separate blood by Ficoll centrifugation, wash twice, count, make sure that cells are HLA-A2 negative (by staining with HB54 and HB117 antibodies)

3. Plate 2×10^6 effectors and 2×10^5 stimulators per well in x24 well plates in culture medium (RPMI 1640, 10% FCS, 1% glutamine, 1% pen/strept 5×10^{-5} M 2-ME) with 500 nM peptide

4. On day 4 prepare stimulators as described in 1. if required. It may be not necessary to preincubate C1RA2 cells for more than 2 hrs

5. On day 5 harvest cells, count spin and seed 5×10^5 effectors, 2×10^6 irradiated (about 3000 rad) HLA-A2 negative autologous feeders, 2×10^5 peptide loaded and irradiated (about 10000 rad) T2 or C1RA2 cells and 500 nM peptide in 2ml of the culture medium with 10u/ml IL-2, 2.5ng/ml IL7 (1/2000 of the stock of 5ug/ml) and with 10% of Q12054 culture sup (anti-CD4 antibodies) (complete medium)

6. 14 days (and further every 2 weeks) later restimulate cultures as described in 5. Cell recovery is usually 200%

7. On day 5 analyse peptide specificity in a ^{51}Cr release assay using T2 or mouse cells transfected with A2 molecule as CTL targets and cold K562 cells as NK targets (10 per 1 labelled target). Use 0.7% TFA for max release instead of 0.5% SDS. Significant peptide specificity is detectable after 5-7 week in culture with E:T ratio 10:1

8. Peptide specific cultures can be cloned seeding 1, 10 and 100 cells/well in round bottom x96 well plates in complete culture medium with 10^4

T2/

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